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Male sterility in grasses of the genus *Lolium*

The invention relates to a method for producing stable male sterile plants of the genus *Lolium* for use in the specific production of hybrid varieties by utilization of heterosis.

Plants, as eukaryotes, have two or more copies of their genetic information per cell. Each gene is usually represented by two alleles, which can be identical in the homozygous condition or different in the heterozygous condition. When two selected inbred lines are crossed, the F1 hybrids produced in the first generation, i.e. heterozygous individuals, are often bigger, more robust and also more productive than the homozygous parents, presumably because both of their allelic gene products a) are less likely to be inactivated, or b) they have a greater reactivity. This effect, called heterosis or hybrid vitality, has already been exploited by plant breeders for many decades for the production of hybrid varieties.

Thus, the phenomenon of heterosis is understood to mean the increase of quantitative feature presentations in progeny beyond the average of the parents or the performance of the superior parent. In particular, the growth (plant length, degree of branching, etc.) and the yield as well as the quantitatively inherited characteristics (for instance resistance) may be affected.

The breeding of hybrid lines is carried out using cytoplasmic male sterility (CMS) or self-incompatibility (SI), the two most important genetic systems for inhibiting self-pollination. A total utilization of heterosis can be achieved by exploiting the cytoplasmic inheritable male sterility (C. Bothe, Nutzung von teilfertilen ms-Linien für die Züchtung von Chance-Hybriden bei Welschem Weidelgras (Lolium multiflorum Lam.), Dissertation Göttingen, Cuvillier Verlag Göttingen 1996, 113 S., G. Kobabe, Heterosis and hybrid seed production in fodder grass, Monographs on Theoretical and Applied Genetics, Vol. 6 Heterosis, Editor: R. Frankel, Springer Verlag Berlin Heidelberg 1983; V. Lein, Heterosis in Kreuzungen zwischen Inzuchtlinien des Deutschen und Welschen Weidelgrases (Lolium perenne L. x Lolium multiflorum Lam. ssp. italicum) Dissertation Göttingen 1988; 91 S.). By one breeding partner losing its ability to produce fertile pollen cytoplasmic male sterility enables the selective production of F_1 hybrids. Female fertility is not affected.

In the breeding of cultivated plants with improved agronomical performance and adapted content, different breeding methods may be used depending on the natural mode of pollination of the respective plant species. While for the strict self-pollinators such as barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) methods of line breeding are used, for obligatory cross-pollinators such as rye (*Secale cereale* L.) population breeding, synthetic breeding and hybrid breeding has been used in the past, for which heterosis is being increasingly used.

The plant material available for breeding possesses a natural variability in the degree of heterosis. A distinction is made here between a general combining ability (GCA) and specific combining ability (SCA). Lines with a high GCA are characterized by a high heterotic performance in breedings with different parent lines. Plant lines with a high SCA show a high heterotic performance in combination with a specific breeding partner. The SCA can therefore only be determined in comparative pairwise breedings (e.g. diallelic).

With respect to the utilization of heterosis, usually plant material is selected for breeding which is characterized by a good natural contribution and a good GCA or SCA.

While for the breeding of population varieties first mass selection, i.e. repeated selection of the best individual plants and joint continuation to a homogenous variety is initially pursued, for the production of so-called synthetic varieties (synthetics) several lines are selected, which are characterized by a good GCA. For seed production, different lines are then selectively cultivated together for two to three generations until they are marketed as seeds. These synthetics usually show a higher degree of heterosis and thus especially a higher yield than the population varieties.

Hybrid varieties are a further progression in the utilization of heterosis in plant varieties. By the selective combination of specific parent lines with good GCA or SCA in the last step of seed production hybrid varieties can be produced which are characterized by a very high heterosis performance and thus a noticeably increased yield.

A precondition for the production of hybrid varieties is the directed pollination of a mother line with a selected father line as pollen donor. In order to produce sufficient amounts of seeds, the latter step has to be performed on large-scale under outdoor conditions. By cultivating a pollenless, i.e. male sterile, mother line and a fertile (pollen-bearing) father line in direct proximity, such a directed pollination is achieved. Thereby hybrid seeds are created to a large extent, which can be solely traced back to the crossing combination of the two parent lines. A precondition for this is particularly the existence of a complete, i.e. if possible 100% male sterile mother line which cannot pollinate itself.

For this reason various methods were developed in the past, in particular mechanical, chemical and genetic methods for the induction of male sterility of plants. Mechanical methods, such as for instance the removal of the anthers, are only suitable for plant species having large and/or spatially separated sexual organs, such as for instance corn (*Zea mays* L.). For the chemical emasculation of plants substances called gametocides were developed, which have a lethal effect on pollen after application. In this way, also hybrid varieties of strict self-pollinators such as wheat and barley could be produced for the first time.

Genetic mechanisms which induce male sterility of plants have been described for a long time. For instance, male sterile plants basically occur in the mostly aneuploid progeny of wide, i.e. inter-specific or intergeneric crossings. This is partly due to irregularities in the meiosis of the progeny and affects both male and female gametes to the same extent. In addition, also systems have been discovered and further refined, which are based on single gene defects and which only influence the male gametes and the pollen. Such systems can be

traced back on the one hand to mutations in the nuclear genome (nuclear male sterility, NMS) and on the other hand to gene alterations in the plastom or cytoplasm (cytoplasmic male sterility, CMS).

The CMS is based in principle on the incompatibility of the nucleus and the cytoplasm and is inherited strictly maternally in most higher plants (U. Witt, Identifikation und Charakterisierung eines kernkodierten Mitochondrienproteins aus dem pollensterilität-induzierenden Polima-Cytoplasma von Brassica napus L., Dissertation, Hamburg 1993).

With the help of a corresponding father line, which is not able to overcome the sterility of the maternal cytoplasm (so-called "maintainer"), theoretically a homozygous sterile CMS plant may be produced after repeated back-crossing with the maintainer plant. A complete CMS system, for example for the production of hybrid seeds of grasses whose vegetative mass is used, thus consists of the following components:
1. the CMS line which bears a sterility-inducing cytoplasm (S), also called sterile mother line.
2. the maintainer line, which bears a normal fertile cytoplasm (N) and which is very similar to the CMS line in other respects.
3. the pollinator line or father line, which is normally fertile and which is suitable for combination with the CMS mother line.

A fundamental technical problem for the production of hybrid varieties is the stability of the male sterility of the CMS line. This particularly affects the 100% transfer of the male sterility to the next generation after crossing and the provision of an environmentally independent phenotype in the form of male sterile plants. Only under these conditions can agronomically optimized and complete male sterile mother plants be generated, which permit heterosis in the form of hybrid varieties to be exploited to its full extent and to realise an additional yield potential.

The *Lolium* species perennial ryegrass (*Lolium perenne* L.), annual ryegrass (*Lolium multiflorum* L.) and hybrid ryegrass (*Lolium hybridum* L.) are the most important grass species in European food grass culture. For food grasses the specific exploitation of heterosis effects is thought to be a real possibility for substantially increasing yield potential and for improving further quantitative characteristics such as stress tolerances against biotic and abiotic factors. As the aforementioned *Lolium* species are cross-pollinators, the breeding of synthetics and hybrid varieties presents itself for this purpose.

In order to achieve additional variability as a basis for the selection of new genotypes, the method of polyploidisation is used in the breeding of cultured plants. With polyploidisation, by using mitosis inhibitors such as colchicin during mitosis the chromosome set of a cell is doubled. In the case of *Lolium* species this leads to the generation of tetraploid forms from originally diploid species ($2n = 2x = 14$), which have a double chromosome set ($2n = 4x = 28$). Because tetraploids possess other characteristics besides diploids, for the economically relevant *Lolium* species *L. perenne, L. multiflorum and L. hybridum* corresponding tetraploid varieties have been cultivated.

For ryegrass species there are a number of studies which point to heterosis and hybrid growth in all valences (including C.A. Foster, Interpopulational and intervarietal hybridisation in Lolium perenne breeding, heterosis under noncompetitive conditions, J. Agric. Sci. 1971, 107-130; C.A. Foster, (1973): Interpopulational and intervarietal F_1-Hybrids in Lolium perenne: performance in field sward conditions, J. Agric. Sci. 1973, 80, 463-477; I. Rod, Beitrag zu den methodischen Fragen der Heterosiszüchtung bei Futtergräsern, Ber. Arbeitstagung Arbeitsgemeinsch. Saatzuchtleiter Gumpenstein 1965, pages 235-252; I. Rod, Remarks on heterosis with grasses, Heterosis in plant breeding, Proc. 7th Congr. Eucarpia Budapest (1967), pages 227-235; A.J. Wright, A theoretical appraisal of relative merits of 50% hybrid and synthetic, J. Agric. Sci. 79, 1972, pages 245-247). In the past, heterosis

effects could be detected especially after single plant crossings, line crossings and variety crossings (Kobabe, see above).

The breeding method most commonly used at present, namely the production of synthetics or varieties on the basis of clones or populations, was developed for grasses by Frandsen (N.H. Frandsen, Some breeding experiments with timothy, Imp. Agric. Bur. Joint Publ. 1940, 3, 80-92) in 1940. However, as mentioned above, this method only allows a partial use of heterosis. A true food grass hybrid variety by using a *Lolium* line with cytoplasmic male sterility for the complete utilization of heterosis (C. Bothe, see above; G. Kobabe, see above; V. Lein, see above) is not known so far, because no plants with complete male sterility were available and the known CMS sources are unstable.

The systems found or used for *Lolium* species for the achievement of male sterility differ with respect to their origin and mode of action. Systems with mechanical control for the castration of the plants are ruled out for *Lolium* species due to their morphology. Chemical methods have not yet been developed for *Lolium*, while genetic control mechanisms were described a long time ago. Spontaneously generated sources have been reported by Nitzsche (Cytoplasmatische männliche Sterilität bei Weidelgras (*Lolium ssp.*) Z. Pflanzenzücht., Berlin (West) 65, (1971), pages 206-220) for *Lolium multiflorum*, and, for *Lolium perenne,* by Gaue (Möglichkeiten der Hybridzüchtung auf ms-Basis bei *Lolium perenne* L. XIII. Internat. Grasland-Kongreß, Leipzig 1977, Sektionsvortrag 1-2, pages 491-496; Ergebnisse von Untersuchungen zur Hybridzüchtung bei *Lolium perenne* Tag.-Ber., Akad. Landwirtsch.-Wiss. DDR, Berlin (1981) 191, pages 119-126). After species and genus crossings male sterile forms also developed for *Lolium perenne* (F. Wit, Cytoplasmic male sterility in ryegrasses (*Lolium* ssp.) detected after intergeneric hybridisation, Euphytica 1974, 23, 31-38; V. Connoly, Hybrid grasses varieties for the future Farm Food Res. 1978, 9, 6, 131-132; V. Connoly, R. Wright-Turner, Induction of cytoplasmic male-sterility into ryegrass (*Lolium perenne*), Theor. Appl. Genet. 1984, 68, 449-453). However, none of these genetic systems

could be stabilized genotypically and phenotypically, so that up to now no functional hybrid system is known for the different ryegrass species.

Although the production of hybrid lines with improved agronomic characteristics is intensively studied, methods available so far for the production of male sterile plants do not lead to completely satisfactory results in many cases. There is therefore a strong need for a method for the production of completely male sterile and stable plants which do not show the disadvantages of the prior art.

It is therefore an object of the present invention to provide methods for the production of stable, i.e. among other things, environmentally independent and completely, i.e. 100%, male sterile plants of the genus *Lolium*, which enable the specific production of hybrid plants and thus utilization of the effects of heterosis such as for example additional yield.

This and other objects of the invention are solved by the provision of embodiments characterized in the patent claims.

According to the invention a method is provided by which it is possible for the first time to produce completely, i.e. 100% male sterile plants of the genus *Lolium* (hereinafter also referred to as MSL or MSL plant (male sterility *Lolium*). This completely new plant of the genus *Lolium* is characterized by a high stability of the sterility-inducing plasm. In particular, the male sterility in the plant produced by the method according to the invention is temperature-stable and shows only an extremely low environmental dependency. In contrast to the sterile crossing progeny of plants with male sterility known up to now, in which, depending on the anther shape, a high degree of partially sterile plants is present, the degree of sterility in MSL is uniformly high, irrespective of the anther shaping (Tables 2 to 4). The MSL lines produced by the method according to the invention consequently for the first time enable the production of homogenous *Lolium*-F_1 hybrid varieties under outdoor conditions.

The method according to the invention for the production of completely male sterile plants of the genus *Lolium* comprises the following steps:

a) mutagenesis of seed material of wild-type plants of the genus *Lolium*;
b) optionally, examination of the mutagenized *Lolium* plants with test methods directed to the pollen vitality and/or by molecular biological methods; and
c) identification of completely male sterile *Lolium* plants.

Within the scope of the invention, wild-type plants are understood to mean naturally occurring plants of the genus *Lolium*, especially those whose genetic information has not been manipulated by mutagenesis. The seed material is especially caryopses material.

The mutagenesis in step a) of the method according to the invention is preferably performed by treatment of the seed material with chemical mutagens. N-ethyl urea is especially preferred for this purpose. Other possible mutagens are alkylating agents such as methylmethanesulfonate and diepoxy butane, urethane, nitroso compounds, alkaloids such as colchicin, peroxides such as H_2O_2, alkyl peroxides and the like.

In an alternative embodiment mutagenesis is carried out by irradiation of seed material with shortwave UV light (e.g. 254 nm); longwave UV light (e.g. 300 to 400 nm) in combination with psoralens; ionising radiation such as X-ray and γ irradiation; and the like.

The *Lolium* plants, in which the cytoplasmic male sterility is produced, are preferably selected from the group consisting of *Lolium perenne, Lolium multiflorum* and *Lolium hybridum*.

The analyis of mutagenized *Lolium* plants using test methods directed to the pollen vitality serves the purpose of differentiating between fertile pollen and the desired sterile pollen.

Because of the good correspondence between anther shape and degree of sterility – 99% of the plants produced by the method according to the invention which were visually classified as sterile, are indeed completely sterile –analyses of pollen vitality are only necessary in the method according to the invention if a control is desired. For the plants with male sterility known from the prior art no reliable visual evaluation of the male sterility produced is possible, because anther shape and degree of sterility do not correspond, or correspond only to a lesser extent (Table 3).

Preferably, the test methods are staining methods, such as for example the method according to Alexander (M.P. Alexander, Differential staining of aborted and nonaborted pollen, Stain Technology 1969, 44/3, 117-122), the addition of light green reagent (I. Šinska, Ergebnisse der Forschung der Pollensterilität der Luzerne d'Eucarpia-Groupe Medicago sativa Piestany 17.-21.5.1976) and the addition of Lugol's solution. The corresponding reagents for the above-mentioned staining methods are shown in Table 1.

Alternatively, or in addition to the above-described analyses of the mutagenized *Lolium* plants with respect to pollen vitality, for example by visual investigation or staining methods, molecular biological methods for the reliable genotypic differentiation between MSL and *Lolium* plants which do not show complete male sterility may be used. Especially preferred molecular biological differentiation is performed by Southern Blot techniques.

Primer pairs for the amplification of the probes used for the Southern Blot hybridisation are preferably selected from the group consisting of the following primer pairs (see also Table 6):

a) TTACTTCACATAGCTTTTCGTU and
CCACAAACCACAAGGATATAG;

b) CGTAAAGGCATGATTAGTTCC and
GATTGTTCTAAAATGGTTATTCCTC;

c) ATGATTGAATCTCAGAGGCAT and
CATATACCTCCCCACCAATAG;

d) TTAGTAGATCGTGAGTGGGTC and
GTGCTAAAAATCCGGTACAT;

e) TTATCCGTCGCTACGCTGTTC and
AATGGAAAGATCGGAACATGG;

f) ATGTTTCCACTCAATTTTCAT and
GCTCCACAGTGGTAAAGTCT;

g) TTACGACCACTGAACAAACTT and
TTTAACCATAAAATCGATTATGC;

h) CTATATTTCGTACGTTTCGGA and
TTATTATGGTAAATTTGTGTATCAA;

i) ATGACTATAAGGAACCAACGA and
GATCAGTCTCATCCGTGTAA;

j) ATGAGACGACTTTTTCTTGAA and
CTTGTAAACTAATCGAGACCG;

k) primer pairs according to the primer pairs shown in a) to j), wherein the corresponding primer sequences differ from the sequences shown in a) to j) by a maximum of 3 bases each, as well as

l) primer pairs corresponding to the primer pairs shown in a) to k), wherein the primer sequences comprise the sequences shown in a) to k),

wherein the sequences are shown in 5'-3' direction and the first sequence is the upper primer.

Restriction enzymes preferred for the Southern Blot hybridisation in the method according to the invention are selected from the group consisting of *Hin*dIII, *Xba*I, *Dra*I, *Eco*RV, *Bam*HI and *Hae*III.

By combining the above-described probes and enzymes, particularly by combination of the probe *nad*9 (see Table 6) with the restriction enzyme *Dra*I, it is possible to clearly distinguish between plants with stable and instable male sterility and complete and incomplete male sterility, respectively.

In another essential aspect of the present invention, plants of the genus *Lolium* with complete male sterility are provided, which can be produced according to the method described above.

A further subject of the invention is a method for the production of stable F_1 hybrids of completely male sterile plants of the corresponding *Lolium* species, comprising the following steps:

a) producing completely male sterile plants of the corresponding *Lolium* species (MSL plants) according to the method described above, and

b) back-crossing the MSL plants obtained from step a) with plants of the same *Lolium* species, which carry a normal fertile cytoplasm and which maintain the sterility of the MSL plants (maintainer plants).

In natural *Lolium* populations a differentiated proportion of maintainer plants is present, which is determined by corresponding test crossings with investigation of the F_1 generation on sterility.

A stable MSL line is preferably obtained by repeated back-crossing with maintainer lines.

In a further preferred embodiment of the present invention, the sterility-inducing plasm of the diploid MSL line is brought to the tetraploid valence by polyploidisation. The feature presentation on the tetraploid valence is analogous to the diploid valence.

The polyploidisation is achieved by treatment of MSL plants with mitosis inhibitors such as for example chalones, colchicin, narcotin, quinones and the like. Especially preferred, the polyploidisation is achieved by treatment with colchicin.

For example, in *L. perenne* and *L. multiflorum* there are both tetraploid and diploid forms. Because the species *L. hybridum* is a direct crossing of the two aforementioned species, the MSL system can also be used in both valences in this species.

Also subject of the present invention are stable MSL lines of completely male sterile plants of the genus *Lolium*, which have been produced by the method described above.

Furthermore, a transfer of the *Lolium perenne* MSL plasm to *Lolium* species such as *Lolium hybridum* and *Lolium multiflorum* is possible by crossings. The present invention thus provides the possibility of producing hybrid plants of the *Lolium* species described above on the basis of a *Lolium* line with stable and complete male sterility.

Both maintainer, MSL line and pollinator (= fertile father line which is used for the production of F_1 hybrids) are preferably of the same species for *L. perenne* and *L. multiflorum*. Because *L. hybridum* per se is a combination of the two first-mentioned species, all three components (maintainer, MSL line and pollinator) may be combined from the two basic species. Maintainers may be isolated or developed by conventional techniques from commercially available varieties and breeding strains. Any pollinator may be selected, because the fertility of the F_1 hybrids is not important, because only the vegetative mass of food grasses is used.

Using the method according to the invention MSL lines could thus be produced, which demonstrate a high degree of male sterility and stability in comparison to male sterile *Lolium* plants hitherto known and which thus differ substantially from these. With the help of the

MSL lines according to the invention it is therefore possible for the first time to produce F_1 hybrid varieties of *Lolium* species, particularly the food grass species *Lolium perenne*, *Lolium multiflorum* and *Lolium hybridum*.

Further, the present invention provides for the first time a method by which the MSL plants may be distinguished from *Lolium* plants with partial or instable male sterility by Southern Blot hybridisation based on the hybridisation pattern.

The present invention is illustrated in the following examples, without limiting it in any way.

Examples

Example 1:

Mutagenesis of the caryopses material of *Lolium* plants

The starting material for the experimental mutagenesis was a diploid *L. perenne* wild-type. The caryopses were incubated using the mutagenic agent N-ethyl urea ($C_3H_8N_2O$) in a concentration of 0.025% for 18 hours.

From the 20,000 *L. perenne* individual seeds treated in this way, 1200 potentially mutated individual plants (first generation after mutagenesis = M_1) could be raised. The remaining caryopses either did not germinate or the shoots were not viable.

The 1200 M_1 plants were then visually inspected for pollen distribution, whereupon 20 individual plants were at first classified as sterile. The individual plants preselected in this way were then tested by three different test methods directed to the pollen vitality (see Table 1): 1. Method according to Alexander (see above), 2. Light green reagent (Šinska, see above),

3. Lugol's solution. After examination 19 individual plants were classified as partially sterile and one single plant (M 361) was classified as completely sterile.

Based on the completely sterile mutant M 361 a stable MSL line in diploid perennial ryegrass, hereinafter referred to as MSL-19, was developed by back-crossing with maintainer lines.

After polyploidisation the cytoplasmic male sterility could also be established in the tetraploid valence. The tetraploid MSL line is hereinafter referred to as MSL-163.

By repeated back-crossing, the transfer of the MSL plasm from *L. perenne* to *L. multiflorum* could be achieved. Here too, MSL material was produced in diploid and tetraploid valence.

Example 2:

Inspection of the pollen of the plants (Pollenbonitur)

The characterisation of the male sterility of the MSL line according to the invention with complete cytoplasmic male sterility was performed by inspection of the anthers and pollen and in comparison with already known CMS (cytoplasmic male sterility) plants of *Lolium perenne*. As a direct comparison first the *L. perenne* CMS line, CMS-1, was used (D. Burkert, R. Schlenker, Pollensterilität bei Lolium perenne L. und Festuca pratensis Hunds. Wiss. Z. Univ. Rostock math.-naturwiss. R., 1975, 4.7, 845-850; I. Gaue, Ergebnisse von Untersuchungen zur Hybridzüchtung bei Lolium perenne Tag.-Ber., Akad. Landwirtsch.-Wiss. DDR, Berlin (1981) 191, 119-126).

Surprisingly, the degree of sterility of the MSL line produced by the method according to the invention was uniformly high, irrespective of the anther shaping of the individual MSL plants, wherein additionally reliable transmission of the sterility-inducing plasm was found for MSL

(see Tables 2 to 4). This distinguishes them from the sterile crossing progeny of CMS sources known from the prior art.

MSL-19 was characterised by a reduced anther size without viable pollen. The latter was also proven by the mentioned staining methods. The feature characteristic of MSL on the tetraploid valence (MSL-163) was analogous to the diploid valence.

Besides the CMS source CMS-1, the variety Inca (CMS-I1 to -I4), which is based on a CMS system, as well as plants of seven CMS sources of the Landessaatzuchtanstalt Hohenheim (CMS-112, CMS-113, CMS-114, CMS-115, CMS-117, CMS-118, CMS-119) were included in the further investigations in order to facilitate a comparison with other available *Lolium* CMS plants. For that, pollen of mature anthers of the individual plants CMS-1 (S), CMS-1 (N) as well as, representing MSL, the individual plants of the line MSL-163 (S) were analysed and compared with each other under two different environmental conditions, i.e. hothouse and outdoor. Moreover, the pollen of the individual plants CMS-112 to CMS-115 as well as CMS-117 to CMS-119 was inspected.

a) CMS-1 (S)

A total of 40 individual plants CMS-1 (S) were investigated. It was shown that besides completely sterile plants, also semi-sterile with percentages of fertile pollen between 20% and 75% (Figure 1A) as well as completely fertile plants (Figure 1B) were present.

The different ratios between fertile and sterile pollen in semi-sterile plants are most probably due to environmental factors, because individual plants produce different percentages of fertile pollen under different environmental conditions. The latter confirms the instability of the source CMS-1.

b) CMS-1 (N)

Of 34 individual plants CMS-1 (N) examined, 32 were classified as completely fertile, 2 individual plants in contrast were semi-sterile, i.e. in squeeze preparation both fertile and sterile pollen could be detected.

c) MSL-163 (S)

Within MSL-163 (S) 20 individual plants were analysed and all were classified as completely sterile. The stability of the CMS in this line could be due to an early termination of the pollen development; stainable pollen grains were not observed in any case.

d) CMS-112 to -115, CMS-117 to CMS-119

The pollen inspection of individual plants of each source showed that the pollen of the sources CMS-112, CMS-113, CMS-114, CMS-115, CMS-117, CMS-118 and CMS-119 should be classified as semi-sterile, i.e. there were stainable fertile pollen grains besides sterile pollen grains in all plants. This can be explained, as in the case of CMS-1 (S), by an instability of the cytoplasmic male sterility.

Example 3:

Molecular biological analyses of MSL plants and comparative plants with cytoplasmic male sterility

In addition to the phenotypical data (pollen inspection of Example 2), the method of Southern hybridisation was used for the reliable genotypical differentiation between MSL and known CMS systems in *Lolium*.

a) Isolation of total DNA

The isolation of total DNA was performed essentially according to Wilkie (Isolation of total genomic DNA. In: M.S. Clark (Ed.) Plant Molecular Biology – A Laboratory Manual. 1997, Springer Verlag, Berlin Heidelberg New York, 3-14). In a stainless steel cylinder pre-cooled with liquid nitrogen (LN_2) 1 to 2 g of LN_2 frozen leaf material was ground with a ball mixer mill (Retsch, Haan) to a fine powder and transferred into 50 ml reaction vessels. It was then incubated in 20 ml 2x CTAB buffer in a water bath at 65°C for 90 min, followed by two extractions with chloroform/isoamylalcohol (24:1, v/v). After centrifugation at 5000 x g for 15 min in a fixed-angle rotor HFA 13.50 (Heraeus, Hanau), the aqueous phase was transferred into new 50 ml reaction vessels. The RNA was degraded by adding 1/100 vol. RNase solution (10 mg/ml) and incubation for 30 min at 37°C. The DNA was precipitated by adding 0.7 vol.-% isopropanol and incubation at room temperature for 20 min. The DNA was transferred into 10 ml 76% ethanol/0,2 M NaOAc with the help of a Pasteur pipette and incubated for 20 min on ice. This was followed by a further washing step for 5 min in 2 ml 70% ethanol on ice. The DNA was then pelleted by centrifugation at 13000 x g for 4 min (Biofuge 22 R, Heraeus). After aspiration of the excess EtOH the pellet was dried at room temperature and dissolved in TE buffer (volume depends on the pellet size). The DNA concentration was determined photometrically at 260 nm (GeneQuant II, Pharmacia Biotech, Freiburg). All buffers used for DNA isolation are shown in Appendix A.

b) Isolation of mitochondrial DNA

Mitochondrial (mt) DNA was isolated according to the protocols of Kiang et al. (Cytoplasmic male sterility (CMS) in *Lolium perenne* L.: 1. Development of a diagnostic probe for the male-sterile cytoplasm. Theor Appl Genet. 1993, 86, 781-787) and Chase and Pring (Properties of the linear N1 and N2 plasmid-like DNAs from mitochondria of cytoplasmic male-sterile *Sorghum bicolor*. Plant Mol Biol 1986, 6, 53-64). As starting material fresh leaf mass of hothouse plants was used that had been darkened before for 16-20 h with black foil. All working steps up to the lysis of the mitochondria were performed at 4°C. In the first working step 60 g of leaf mass were ground in 400 ml extraction buffer in a jug mixer

(Gastronom GT95, W. Krannich, Göttingen), filtered through 5 layers of gauze (YPSIGAZE 8-fold, Holthaus-Medical, Remscheid) and then centrifuged for 10 min at 5000 x g. The supernatant was decanted into a new centrifuge tube and centrifuged again for 10 min at 16000 x g for the pelleting of the mitochondria. The mitochondrial pellet was then resuspended with the help of a sterile hair brush in 8 ml DNAse buffer. After adding 8 mg DNAse I, the nuclear and plastid DNA was degraded (90 min at 4°C). In the next step the mitochondrial suspension was underlaid with 20 ml washing buffer and centrifuged at 12000 x g (30 min). This was followed by a further resuspension of the pellet in washing buffer with subsequent centrifugation.

The mitochondria were lysed in 3 ml lysis buffer to which was added proteinase K (100 µg/ml final concentration) and 0.5% SDS at 37°C (1 h). 3 ml extraction buffer was then added and the samples were incubated at 65°C for 10 min in a water bath.
After aliquoting the samples (600 µl) in Eppendorf reaction vessels (2 ml), the proteins were precipitated by adding 200 µl 5 M potassium acetate on ice (20 min). The proteins were pelleted by centrifugation of the samples for 5 min at 13000 rpm, the supernatants were transferred into new reaction vessels and the mtDNA was precipitated by adding 400 µl isopropanol and 40 µl 5 M ammonium acetate overnight at -20°C. After centrifugation for 5 min at 13000 rpm, the mtDNA was pelleted and resuspended in 100 µl "50-10" TE buffer.

This was followed by the bringing together of several aliquots to a volume of 500 µl and the degradation of RNA by adding RNase A (100 µg/ml final concentration) for 1 h at 37°C. The mtDNA was precipitated again as described above, the DNA pellet was dissolved in 100 µl TE buffer. The concentration was determined as in the case of the total DNA. All buffers used for the isolation of mtDNA are shown in Appendix A.

c) Restriction, electrophoresis and blotting

For the hybridisation experiments, 5 µg of total or mtDNA were restricted with 5 U restriction enzyme, adding the corresponding reaction buffers, for at least 4 h at 37°C. The endonucleases *Hin*dIII, *Bam*HI, *Eco*RV, *Xba*I, *Dra*I and *Hae*III (Gibco BRL, Eggenstein) were used as restriction enzymes.

The DNA fragments were separated in a 1% agarose gel for 6-8 h at 50-60 V in 1xTAE buffer after adding 5 x loading buffer and then stained with an ethidium bromide solution (0.1 mg/ml). The size of the fragments was determined with the aid of a DIG labelled DNA length standard (Roche, Grenzach-Wyhlen).

The DNA was then transferred to a positively loaded nylon membrane (Roche) by capillary blot. The transfer took place overnight, 20 x SSC solution was used as transfer medium.

After DNA transfer the filters were washed in 2xSSC (10 min) and sealed while still moist in plastic foil until hybridisation took place. For fixing the DNA a UV radiation took place (30 s; 0.120 J/cm^2).

d) Description of the probes

The primers for amplification of the gene probes required were derived from cDNA sequences of the mitochondrial genome of *Arabidopsis thaliana* after database searches (EMBL; ID Miatgen). Different genes which code for ribosomal proteins and subunits of the protein complexes of the respiratory chain were selected (see Table 5).

For control of contamination of isolated mtDNA with genomic DNA a ubiquitous nuclear genomic cDNA gene probe (actin) was used. The primers were derived from known actin cDNA sequences from rice; the amplification was performed with rye pollen cDNA.

e) Generation of the probes

The primer pairs for the amplification of the probes used were derived from cDNA sequences of the corresponding genes with the help of the computer programme OLIGO 5.0 (see Table 6). The probes were labelled by incorporation of Digoxigenin-dUTP (Roche) during the PCR reaction with a thermocycler, model UNO from Biometra (Göttingen).

The reaction parameters are shown in Tables 7 and 8.

f) Non-radioactive Southern hybridisation

For the non-radioactive Southern hybridisation the DIG system from Roche was used. The reaction was performed in hybridisation tubes in a hybridisation oven (Stuart Scientific, Staffordshire, UK). 1-2 filters per tube were prehybridised in 20 ml DIG-Easy-Hyb (Roche) for at least 1 h. The prehybridisation solution was then replaced by a new hybridisation solution containing the labelled probe. Prior to adding the probe it was denatured in a water bath (100°C) for 10 min and then incubated for 5 min on ice. 2-5µl labelled probe DNA were used per ml hybridisation solution. The hybridisation was performed for at least 15 h at 39°C. The detection reaction was performed according to Roche protocols. The exposure time of the X-ray films was 10 min to 2 h, depending on the signal strength. The filters were rehybridised according to the manufacturer's instructions. The probe solutions were stored at -20°C and used for another 8 to 10 hybridisations after denaturing at 68°C in a water bath.

Example 4:

Detection of mt-specific signals after hybridisation of total DNA

It was first of all clarified whether using total DNA in combination with mitochondrial probes can lead to other or additional hybridisation signals, which could be due to the presence of mitochondrial sequences in the nuclear genome. For this reason, mitochondrial DNA was isolated from freshly harvested leaf samples of the plants CMS-1 (N), CMS-1 (S),

MSL-163 (N) and MSL-163 (S) to be investigated and the hybridisation results were compared with those using total DNA. After restriction of total DNA and mtDNA of the same line with the restriction enzyme *Hin*dIII and electrophoretic separation of the samples in the agarose gel, no DNA could be visually detected after ethidiumbromide staining. In the lanes with total DNA a continuous fragment distribution in the range of about 1 to 23 kb could be observed, which points to the total restriction of the DNA (see Figure 2A).

After hybridisation with the mt gene probe *nad9* and mtDNA or total DNA as template, identical hybridisation signals could be detected. The result shows that by using mt gene probes and total DNA as template, mtDNA-specific signals can be detected (see Figure 2B).

In comparison thereto, by using a cDNA probe of the nuclear-encoded actin gene it could be shown that the mtDNA was not contaminated with genomic DNA, because as expected, only in the lanes with total DNA hybridisation signals could be detected (see Figure 2C). Because it was thus shown that the probes were mtDNA-specific, total DNA was used in further experiments.

For more detailed molecular biological studies, the plant material was divided into two groups. The first group consisted of the comparative CMS line CMS-1 (S) and the corresponding maintainer CMS-1 (N), the second group included the male sterile MSL lines MSL-163 (S) and MSL-19 (S) and the corresponding male fertile maintainer lines MSL-163 (N) and MSL-19 (N).

A total of 34 different probe/enzyme combinations were tested, 14 of which made it possible to distinguish between the male sterile lines (S) and the corresponding maintainers (N) (see Table 9).

Further, the (S) plasms of the two groups CMS and MSL could be clearly distinguished, especially by using the probe *nad9* in combination with different restriction enzymes (see Table 10).

An extended set of available *Lolium* CMS sources was integrated into the studies with the probe/enzyme combination *nad9/Dra*I: three plants of the CMS-Inca source (CMS-I1, CMS-I2, CMS-I3, CMS-I4), CMS-113, CMS-114, CMS-115, CMS-117, CMS-118 and CMS-119 (see Figure 3). Here, the MSL plants MSL-163 and MSL-19 could be clearly distinguished from all other CMS sources, which for their part showed an identical hybridisation pattern among themselves.

Figures

Figure 1: A, pollen of an individual plant CMS-1 (S) after KES staining; B, pollen of an individual plant CMS-1 (N) after KES staining (magnification scales are not identical).

Figure 2: A, restriction digest of total DNA (a) and mtDNA (b) of the plants MSL-19, MSL-163 and CMS-1 with the restriction enzyme *Hin*dIII; B, Southern hybridisation with the probe/enzyme combination *nad9/Hin*dIII; C, Southern hybridisation with the probe/enzyme combination *Actin/Hin*dIII; (S) = CMS plant; (N) = maintainer.

Figure 3: Southern hybridisation (probe/enzyme combination *nad9/Dra*I) of DNA bulks of the plant Inca (CMS-I1, CMS-I2, CMS-I3, CMS-I4) and CMS-1 (S), MSL-163 (S) and MSL-19 (S) and of individual plants of the sources CMS-113, CMS-114, CMS-115, CMS-117, CMS-118 and CMS-119.

Table 1: Staining methods for the pollen vitality test

Method for the differentiation of fertile and sterile pollen	**(ALEXANDER, 1969)**
Staining:	
fertile pollen grains	dark purple
sterile pollen grains	bright green
Composition of the solution:	
Ethyl alcohol	10ml
Malachite green (1% in 96% alcohol)	1ml
dist. water	50ml
Glycerol	25ml
Phenol	5g
Chloral hydrate water	5g
Fuchsin (1% in water)	5ml
Acid Orange 10 (1% in water)	0.5ml
Glacial acetic acid (pH 3.2)	1-2ml
Light green	**(ŠINSKA, 1976)**
Staining:	
Fertile pollen grains	Dark green with clear netlike surface structure
Sterile pollen grains	Mostly completely colourless, and stained a weak green by degenerated plasma
Composition of the solution :	
Glycerol	1 part
Lactic acid	1 part
Phenol	1 part
Lugol's solution	
Staining:	
Fertile pollen grains	Red staining
Composition of the solution :	
iodine-potassium iodide	

Table 2: Feature presentations of crossing progeny of the CMS line MSL-19 and CMS-1 (both *Lolium perenne*)

Feature	**MSL-19**	**CMS-1**
Valence	Diploid	Diploid
Generation	experimental mutagenesis with N-ethylurea	Spontaneous (found in assortments and propagation stocks in Gülzow in 1969 & 1970; BURKERT and SCHLENKER, 1975)
Visual sterility	very good	very good
Anther expression	white-green-yellow sterile or mixed colours	white-green-yellow sterile or mixed colours
Visual sterility/ degree of sterility	good correspondence independently of anther shaping	No correspondence High degree of partially sterile depending on the anther shaping
Environmental dependence	very low	high

Table 3: Percentage of completely sterile genotypes in F_1 crossing progeny of *Lolium perenne* depending on the CMS source

CMS-Source (S)	**Mode of origin**	**Number of visually sterile F_1 plants examined after backcrossing with maintainer (N)**	**relative percentage of completely sterile plants**
CMS-1	spontaneous	1333	53.3 [x)]
CMS-INCA	Interspecific crossing	172	47.7 [x)]
CMS-5 B (Ireland)	Interspecific crossing	225	77.0 [xx)]
MSL-19	Mutagenesis with N-ethylurea	1500	99.0 [xxx)]

x) Detection staining method light green reagent
xx) According to Conolly (1984) sterility class 1 and 2[v]
xxx) Detection staining method Alexander
v Sterility class 1: Anthers are flat, non-dehiscent, shrunken, mostly white or translucent with thin cell walls. The anthers contain no pollen.
Sterility class 2: Anthers are non-dehiscent and shrunken, but not quite as flat as in class 1, anthers contain no viable pollen, some empty pollen cases are present on staining.

Table 4: Transmission of sterility of *Lolium perenne* CMS lines MSL-19 and CMS-1; results of the sterility test of F_1 material with identical pollinators (maintainers)

Crossing combination CMS (S) x pollinator (N)	**Number of F_1 plants investigated**	**Results of the sterility test (relative values)**	
		visual sterility	**pollen vitality [x]**
MSL-19 x 85/5/9/6	30	100	0
MSL-19 x KE 23/85	35	100	0
CMS-1 x 85/5/9/6	30	100	15.20
CMS-1 x KE 23/85	30	100	13.80

[x] According to staining method of Alexander (see above)

Table 5: Function of the mitochondrial genes used in higher plants

Protein	**Mitochondrial gene**
Subunits of cytochrome C oxidase	*coxI, coxII, coxIII*
NADH dehydrogenase	*nad6*
NADH: Ubiquinone oxidoreductase	*nad9*
Ribosomal proteins: large subunit small subunit	*rpl6* *rps3*
Apocytochrome b	*cob*
Cytochrome C biogenesis ORF 206	*ccb206*

Table 6: Description of the primer pairs used (U = upper primer, L = lower primer)

Probe	**Primer (5'- 3')**		**Annealing temperature**	**Amplicon (bp)**
coxI	TTACTTCACATAGCTTTTCGTU	U	52.1°C	1556
	CCACAAACCACAAGGATATAG	L		
coxII	CGTAAAGGCATGATTAGTTCC	U	52.3°C	697
	GATTGTTCTAAAATGGTTATTCCT C	L		
coxIII	ATGATTGAATCTCAGAGGCAT	U	53.0°C	797
	CATATACCTCCCCACCAATAG	L		
nad6	TTAGTAGATCGTGAGTGGGTC	U	51.6°C	563
	GTGCTAAAAATCCGGTACAT	L		
nad9	TTATCCGTCGCTACGCTGTTC	U	55.0°C	3392
	AATGGAAAGATCGGAACATGG	L		
rpl5	ATGTTTCCACTCAATTTTCAT	U	52.2°C	522
	GCTCCACAGTGGTAAAGTCT	L		
rpl6	TTACGACCACTGAACAAACTT	U	53.0°C	535
	TTTAACCATAAAATCGATTATGC	L		
rps3	CTATATTTCGTACGTTTCGGA	U	52.4°C	1594
	TTATTATGGTAAATTTGTGTATCA A	L		
cob	ATGACTATAAGGAACCAACGA	U	52.1°C	1174
	GATCAGTCTCATCCGTGTAA	L		
ccb206	ATGAGACGACTTTTTCTTGAA	U	52.2°C	616
	CTTGTAAACTAATCGAGACCG	L		
Actin	CACACTGTCCCCATCTATGAA	U	57.9°C	650
	CTCTTGGCTTAGCATTCTTGG	L		

Table 7: PCR conditions for the generation of the DNA probes used

PCR components	**Amount**
DIG dUTP (10x)	5µl
d-NTP-Mix (10mM)	1µl (200µM)
Primer (5µM)	2.5µl each (0,25µM each)
Taq polymerase* (5U/µl)	0.15µl (0,75 U)
10x reaction buffer	5µl
$MgCl_2$ solution (50mM)	1.55µl (1,5µM)
H_2O	17.3µl
Template-DNA	15µl (75ng)
	Reaction volume 50µl

*Silverstar, Eurogentec

Table 8: PCR conditions (30 cycles 2.-4.)

1.	Denaturation (single)	94°C	2min
2.	Denaturation	94°C	1min
3.	Annealing	see Table 6	1min
4.	Extension	72°C	2min
5.	Extension prolongation (single)	72°C	2min

Table 9: Discrimination between (S) and (N) cytoplasm of the plants MSL-19 and MSL-163 (MSL) in comparison to the plant CMS-1 (CMS)

	Restriction enzyme					
mt gene probe	*Hin*dIII MSL CMS	*Xba*I MSL CMS	*Dra*I MSL CMS	*Eco*RV MSL CMS	*Bam*HI MSL CMS	*Hae*III MSL CMS
coxI	+ +	n. d.	+ +	n. d.	+ +	n. d.
coxIII	+ +	n. d.	+ +	+ +	+ +	n. d.
nad6	+ +	n. d.	+ +	- -	+ +	- -
nad9	- +	- +	+ +	- +	+ +	- +
ccb206	- -	- -	- -	+ -	- -	- -
rpl5	- -	n. d.	n. d.	- -	n. d.	n. d.
rpl6	+ -	- -	+ +	n. d.	+ -	n. d.
cob	n. d.	+ -	n. d.	+ -	n. d.	+ -
rps3	n. d.	n. d.	n. d.	+ +	n. d.	n. d.

MSL = DNA bulk of the plants MSL-19 and MSL-163

CMS = male sterile plant CMS-1

\+ = Discrimination between (N)- and (S) cytoplasm is possible

\- = Discrimination between (N)- and (S) cytoplasm is not possible

n. d. = not determined probe/enzyme combinations

Table 10: Hybridisation pattern of the (S) cytoplasms of the plants MSL-19 and MSL-163 (MSL) in comparison to the plant CMS-1 (CMS)

	Restriction enzyme					
mt gene probe	*Hin*dIII MSL CMS	*Xba*I MSL CMS	*Dra*I MSL CMS	*Eco*RV MSL CMS	*Bam*HI MSL CMS	*Hae*III MSL CMS
CoxI	1 2	n. d.	1 2	n. d.	1 1	n. d.
coxIII	1 2	n. d.	1 2	1 2	1 1	n. d.
nad6	1 2	n. d.	1 1	1 1	1 2	1 1
nad9	1 2	1 2	1 2	1 2	1 2	1 2
ccb206	1 1	1 1	1 1	1 2	1 1	1 1
rpl5	1 1	n. d.	n. d.	1 1	n. d.	n. d.
rpl6	1 1	1 1	1 1	n. d.	1 1	n. d.
cob	n. d.	1 2	n. d.	1 1	n. d.	1 2
rps3	n. d.	n. d.	n. d.	1 1	n. d.	n. d.

MSL = Plants MSL-19 and MSL-163

CMS = Male sterile plant CMS-1

n. d. = not determined

Matching numbers within a probe/enzyme combination indicate identical hybridisation patterns.

Appendix A

Isolation of total DNA:

2xCTAB
0.1 M Tris/HCl pH 8.0
1.4 M NaCl
0.5 M EDTA pH 8.0
2% CTAB
0.04 M β-mercaptoethanol

TE buffer
10 mM Tris/HCl pH 8.0
1 mM EDTA pH 8.0

Isolation of mtDNA

Extraction buffer 1
0.44 M Sucrose
50 mM Tris pH 8.0
3 mM EDTA pH 8.0
0.5% BSA
10 mM β-mercaptoethanol

DNase buffer
0.44 M Sucrose
50 mM Tris pH 8.0
10 mM $MgCl_2$

Washing buffer
0.6 M Sucrose
25 mM EDTA pH 8.0
50 mM Tris pH 8.0

Lysis buffer
50 mM Tris
20 mM EDTA
0.5% SDS
pH 8.0

Extraction buffer 2
0.15 M Tris

"50–10" TE buffer
50 mM Tris

0.1 M NaCl
80 mM EDTA
1.5% SDS
pH 8.0

10 mM EDTA
pH 8.0

TE buffer
10 mM TRIS
1 mM EDTA
pH 8.0

Electrophoresis and blotting

1x TAE
40 mM Tris/acetate
1 mM EDTA

20x SSC
3 M NaCl
0.3 M Na-citrate
pH 7.0

5x loading buffer
12.5 % Ficoll
62.5 mM EDTA pH 8.0
0.5 % SDS
5x TAE
0.02 % bromphenol blue
0.02 % xylene cyanol

Claims

1. Method for the production of completely male sterile plants of the genus *Lolium*, comprising the following steps:
a) mutagenesis of caryopses material of wild-type plants of the genus *Lolium*;
b) optionally examination of the mutagenised *Lolium* plants by test methods directed to pollen vitality and/or by molecular biological methods; and
c) identification of completely male sterile *Lolium* plants.

2. Method according to claim 1,
characterized in that the mutagenesis is performed by addition of chemical mutagens, particularly of N-ethyl urea.

3. Method according to claim 1 or 2,
characterized in that the *Lolium* plants are selected from the group consisting of *Lolium perenne, Lolium multiflorum* and *Lolium hybridum.*

4. Method according to any of the preceding claims,
characterized in that the test methods directed to pollen vitality are staining methods.

5. Method according to claim 4,
characterized in that the staining method is selected from the group consisting of the method according to Alexander, the addition of light green reagent as well as the addition of Lugol's solution.

6. Method according to any of the preceding claims,

characterized in that the molecular biological methods for the examination of the mutagenised *Lolium* plants are Southern Blot techniques.

7. Method according to claim 6,

characterized in that the primer pairs for the amplification of the probes used for the Southern Blot hybridisation are selected from the group consisting of the following primer pairs:

a) TTACTTCACATAGCTTTTCGTU
CCACAAACCACAAGGATATAG

b) CGTAAAGGCATGATTAGTTCC
GATTGTTCTAAAATGGTTATTCCTC

c) ATGATTGAATCTCAGAGGCAT
CATATACCTCCCCACCAATAG

d) TTAGTAGATCGTGAGTGGGTC
GTGCTAAAAATCCGGTACAT

e) TTATCCGTCGCTACGCTGTTC
AATGGAAAGATCGGAACATGG

f) ATGTTTCCACTCAATTTTCAT
GCTCCACAGTGGTAAAGTCT

g) TTACGACCACTGAACAAACTT
TTTAACCATAAAATCGATTATGC

h) CTATATTTCGTACGTTTCGGA
TTATTATGGTAAATTTGTGTATCAA

i) ATGACTATAAGGAACCAACGA
GATCAGTCTCATCCGTGTAA

j) ATGAGACGACTTTTTCTTGAA
CTTGTAAACTAATCGAGACCG,

k) primer pairs according to the primer pairs shown in a) to j), wherein the corresponding primer sequences differ from the sequences shown in a) to j) by a maximum of 3 bases each, as well as

l) primer pairs corresponding to the primer pairs shown in a) to k), wherein the primer sequences comprise the sequences shown in a) to k),

wherein the sequences are shown in 5'-3' direction and the first sequence is the upper primer.

8. Method according to claim 6 or 7,

characterized in that the restriction enzymes are selected from the group consisting of *Hin*dIII, *Xba*I, *Dra*I, *Eco*RV, *Bam*HI and *Hae*III.

9. Plants of the genus *Lolium* with complete male sterility,

produced according to the method according to any of the preceding claims.

10. Method for the production of stable F_1 hybrids of completely male sterile plants of the genus *Lolium*, comprising the following steps:

a) production of completely male sterile plants of the genus *Lolium* (MSL plants) according to the method according to any of claims 1 to 11, and

b) back-crossing the MSL plants obtained in step a) with plants of the genus *Lolium*, which have a normal fertile cytoplasm and which maintain the sterility of the MSL plants (maintainer plants).

11. Method according to claim 10,

characterized in that plants of the corresponding species are used as maintainer plants, **which lead to a 100% pollen-sterile progeny after crossing with the MSL line.**

12. Method according to claim 10 or 11,

characterized in that a multiple back-crossing with maintainer plants is performed.

13. Method according to any of claims 10 to 12,
characterized in that the sterility-inducing plasm of the MSL plant produced in step a) is brought to a preferably tetraploid valence by polyploidisation.

14. Method according to claim 13,
characterized in that the polyploidisation is achieved by treatment with colchicine.

15. Plants of the genus *Lolium* with complete male sterility, produced according to the method according to any of claims 12 to 14.

16. Use of the completely male sterile plants of the genus *Lolium* according to claim 15 for the production of hybrids with pollinator plants having normal male fertility.

SUMMARY

The invention relates to a method for producing stable male sterile plants of the genus *Lolium* and the use thereof in the specific production of hybrid varieties by utilization of heterosis.